Vol. 87, No. 2 October, 1944

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

BIOCHEMICAL FACTORS IN THE MAXIMAL GROWTH OF TETRAHYMENA[1]

VIRGINIA C. DEWEY[2]

Arnold Biological Laboratories, Brown University, Providence, Rhode Island

Introduction

The earliest attempt to elucidate the nutritional requirements of the ciliate *Tetrahymena geleii* (*Glaucoma piriformis*) by the pure culture technique was made by Lwoff (1924). At that time the failure of the ciliate to grow in solutions of pure amino acids was attributed to a lack of specific chemical supplements. Later Lwoff (1932) suggested that a requirement for polypeptides was responsible for the lack of growth in such media.

This early work indicates immediately that the problem of the nutrition of *T. geleii* is a dual one. No investigation of the nitrogen requirements may be made without some knowledge of the supplementary factors needed. So far it has been shown that thiamine is important if not absolutely necessary to the nutrition of *T. geleii,* while riboflavin, pantothenic acid, nicotinic acid and pyridoxine probably play a part (Elliott, 1935b, 1939; Lwoff and Lwoff, 1937, 1938; Hall, 1940a, b, 1942; Baker and Johnson, 1941; Kidder and Dewey, 1942). In a preliminary report Dewey (1941) indicated that other factors of unknown nature are required for maximal growth. A great deal less work has been done on the nitrogen nutrition (Elliott, 1935a; Hall and Elliott, 1935; Dewey, 1941; Hall, 1942). None of the results of these investigations is conclusive. The report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in an amino acid solu- requires confirmation.

An attempt to obtain knowledge of the supplementary requirements necessitates the use of a basic medium capable of supplying the nitrogen and carbon needs of the organism and ideally completely lacking in supplementary factors; the testing of all known growth promoting substances, and the search for and purification of possible unknown growth factors.

I take pleasure in acknowledging my indebtedness to Professor G. W. Kidder of Brown University for his invaluable advice and encouragement. Thanks are due to Dr. R. J. Williams for a gift of pure calcium pantothenate and to Dr. Samuel Lepkovsky for the gift of a concentrate of his factor I.

[1] Presented to the Graduate Council of Brown University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Aided by grants from the Manufacturers' Research Fund for Bacteriology and Protozoology and from the Miss Abbott's School Alumnae Fellowship Fund at Brown University.

Material and Methods

Preliminary experiments were carried out using four strains of *Tetrahymena geleii* and one strain of *T. vorax*. Since only quantitative differences were found between these strains, later work was confined to strain W (Claff, 1940; Kidder, 1941a). This strain was selected because it showed the most rapid growth rate and the greatest resistance to increased salt concentration of the medium. Certain experiments were also carried out using strain H, obtained from Dr. R. H. Hall.

All experimental media were prepared with water distilled twice in an all-Pyrex still over potassium permanganate and all the glassware used was Pyrex. Glassware was cleaned by soaking for at least one hour in a hot saturated solution of trisodium phosphate or a preparation sold commercially as "Keego," followed by careful rinsing to remove all traces of the cleaning agent.

Stock cultures were maintained in a 2 per cent solution of Difco proteose-peptone and also for a time in a one per cent solution of crude casein (Eimer and Amend). The fact that these ciliates could be maintained in solutions of crude casein suggested the use of a highly purified casein as a basic medium. Casein Harris (highest chemical purity) was used as a one per cent solution. Although this preparation has been called "vitamin-free" casein, this term is somewhat misleading in that traces of certain growth substances appear to be present in amounts sufficient to affect the growth of protozoa. It is, however, still adequate as a basic medium for testing responses to growth stimulants because the growth in it alone is still far from optimal with regard either to the rate of growth or the size of the population supported. For the purposes of this paper the growth in crude casein is regarded as being optimal.

Since casein, in order to be available to the ciliates, must be in solution or at least colloidal suspension, the following method was used to disperse it. To the casein was added sufficient alcohol to completely wet it, then water containing 1.0 to 1.5 ml. of normal NaOH per gram of casein was poured in slowly with stirring. With vitamin-free casein it was found necessary to add a balanced salt solution in order to obtain growth. For this purpose the modified Osterhout solution employed by Barker and Taylor (1931) was used in all but the first series of experiments. The concentrated stock solutions were added to the water used to make up the medium. The suspension of casein was allowed to stand with occasional stirring until solution was complete. Then normal HCl was added drop by drop with stirring between drops until the alkali had been neutralized. Care must be taken to add the acid slowly in order to prevent precipitation of the casein. The reaction of the medium was adjusted to pH 6.8–pH 7.0. The medium was then dispensed into tubes in amounts of 5 ml., plugged with cotton and autoclaved for 15 min. at 15 lbs. pressure. Elliott's (1939) report that vitamin-free casein required digestion with pepsin before it could be utilized by the ciliates is possibly due to a failure to put the casein into solution.

Experimental cultures were examined for growth, the results recorded and transplants made at intervals of 48 hours in casein media. The interval was slightly longer in gelatin media. The tubes were kept, however, for from one to two weeks and re-examined at intervals. Such a procedure gives results which indicate the presence or absence of factors necessary to maintain growth at or near a maximum rate. These results cannot, of course, be compared with those obtained by in-

cubating cultures for a week or more before examination, since by that time a slowly growing culture may have reached a concentration equal to that of a rapidly growing culture. In order to eliminate the effects of carry-over only the results of the third transplant in a given medium are considered. Growth is recorded as zero to four plus by comparison with growth in a control medium. Growth recorded as zero may indicate survival of the inoculum or an increase of one or two divisions, while four plus growth represents a population of from 75,000 to 100,000 organisms per ml. Two or three plus growth is intermediate. The cultures were kept at room temperature (20°–22° C.). Transplants were made using an open bacteriological loop and ordinary bacteriological technique.

Cultures were also incubated in Kidder culture flasks. The third transplant in a tube was inoculated into the flask and the growth followed by making counts at intervals of 12 or 24 hours (Kidder, 1941a).

Gelatin in concentrations of 1 per cent, 1.5 per cent and 2 per cent was also used in certain experiments. Both Harris gelatin (vitamin-free) and Eastman de-ashed gelatin were used. Another medium consisted of 1 per cent silk peptone (Seidenpepton, Hoffman-LeRoche). These media were in some cases supplemented with amino acids in various concentrations as well as with various growth promoting substances. In other cases solutions of the pure amino acids alone were used. The following amino acids were obtained from the Eastman Kodak Co.: l-histidine, l-leucine, dl-threonine, dl-β-phenylalanine, dl-methionine, d-arginine carbonate and d-lysine hydrochloride. From the Hoffman-LaRoche Co. l-tryptophane, d-isoleucine, dl-valine and glycine were obtained and from Eimer and Amend, tyrosine.

The basic medium was supplemented with vitamins and growth factors of known chemical composition as well as with crude extracts of animal and plant material. The known compounds were supplied (with one exception to be noted later) at a level of 0.001 mg. per ml. except in the case of i-inositol, which was used in a concentration of 0.004 mg. per ml., and biotin, which was used in a concentration of 0.00008 mg. per ml. Thiamine hydrochloride and riboflavin were obtained from the Hoffman-LaRoche Co. A sample of calcium pantothenate was obtained from Dr. R. J. Williams and subsequent calcium pantothenate as well as biotin methyl ester from the S. M. A. Corp. Pyridoxin hydrochloride (first used as factor I concentrate of Lepkovsky) was obtained from Merck and Co. and nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid from the Eastman Kodak Co.

Water extracts of crude casein, egg yolk, yeast (Harris), timothy hay and alfalfa meal (Denver Milling Co.) were also used. Only the last two were used in routine culturing and in experiments on fractionation. The extracts were prepared by boiling 50 g. of material with a liter of water for ten min. and filtering with suction using Celite, analytical grade (Johns-Manville), as a filter aid. The timothy extract was used in a dilution of 1 : 5 and the alfalfa extract in a dilution of 1 : 10.

The crude extracts were treated in various ways in an attempt to remove the protein present as a preliminary to a study of the nitrogen requirements of *T. geleii*. The results of these fractionations were so interesting that studies on them were continued while the work on the nitrogenous nutrition was in progress. Tests for protein or its degradation products were made by the ninhydrin reaction.

One of the first methods tried for the removal of protein was precipitation with

lead acetate. A 25 per cent solution of normal lead acetate was added to the extract until precipitation was complete. The precipitate was then filtered off with the aid of suction and Celite. Excess lead was removed from the filtrate and the precipitate was decomposed by the use of phosphate. In the first experiments a 5 per cent solution of phosphoric acid was used but later a saturated solution of trisodium phosphate was found to give better results. The precipitated lead phosphate was then removed by filtration with suction. A similar technique was used in the preparation of the fractions obtained with ferric oxide hydrosol (prepared according to the method of Thomas and Frieden, 1923).

Barium hydroxide was used as a precipitant after the addition of three volumes of alcohol to the extract. This latter step was necessary because barium hydroxide alone caused little precipitation when added to the aqueous extract. In this case barium was removed by the use of sulfuric acid and the alcohol by boiling. When phosphotungstic acid was used the extract was first made acid by the addition of sulfuric acid to give a concentration of 50 per cent. The sulfuric acid alone caused the formation of a precipitate which was removed by filtration before the addition of the phosphotungstic acid. After separation of the phosphotungstic acid precipitate both the filtrate and the precipitate were treated with barium hydroxide to remove the sulfuric acid and the phosphotungstic acid.

The method given by Peters and Van Slyke (1931) for the removal of carbohydrate by the use of copper sulfate and calcium hydroxide was tried and found to remove all the reducing sugars present in the extracts, although protein was not removed. Excess calcium was removed from the filtrate either as the carbonate or the phosphate.

Precipitation was also carried out by the use of alcohol or acetone. After adding sufficient alcohol or acetone to give the desired concentration the extracts were allowed to stand until flocculation was complete. The precipitates were then filtered off. To remove the precipitant the filtrates were boiled or distilled, sometimes under reduced pressure. The precipitates were redissolved in water. The above named solvents as well as ether or acetic acid were also used to make extracts of alfalfa meal, using a Soxhlet apparatus except in the case of the acetic acid. The solvents were removed from the extracts by boiling and the residues taken up in water.

When it was found that the active material in these extracts was adsorbed on charcoal (Norit) and to some extent on Super Filtrol (Filtrol Corp.), attempts were made to obtain elution. Various concentrations of methyl and ethyl alcohols at various pH's were tested. The most successful eluting agent consisted of 50 per cent ethyl alcohol containing 10 per cent ammonium hydroxide. Both of these substances could be removed from the eluates by boiling.

Dialysis of the extracts was carried out in cellophane against distilled water. During the process, which lasted for several days (changing the water outside the cellophane at intervals of 12 hours), a temperature of from 50° to 60° C. was maintained in order to prevent bacterial action. An electric light bulb was used to heat the box in which dialysis was carried out. The diffusate and the dialysate were boiled down or made up to the original volume.

The active materials were tested for stability by adjusting the pH of portions of the extracts to values ranging from pH 3.0 to pH 10.0 and then heating in the autoclave at 15 lbs. pressure for one hour. Extracts were also boiled for 24 hours

in the presence of five per cent sulfuric acid in an attempt to remove tannins (Harrison and Roberts, 1939). The results gave an additional test of stability to acid.

In all cases the pH of the extracts or of the fractions was adjusted to approximate neutrality in order to avoid changing the pH of the medium or precipitation of the casein upon addition of the extract.

Results

A. Supplementary Factors

None of the strains of Tetrahymena gave growth in the vitamin-free casein medium alone or with the addition of various known growth factors. Upon the addition of 0.08 per cent of a water extract of yeast (Harris) or a water extract of crude casein good growth was obtained. This demonstrated that the casein was available to the ciliates and that the failure to grow was due to a lack of some substance, although the possibility that some toxic substance was neutralized should be kept in mind.

It was then found that when the basic medium was made up with the inorganic salt solution of Barker and Taylor growth of the ciliates occurred in the third transplant without the addition of any supplement. This growth was, however, extremely slow, taking a week or ten days to reach a maximum density of about 1000 organisms per ml., which was far below that in controls in crude casein or vitamin-free casein supplemented with hay or alfalfa extract. Addition to the basic medium of thiamine, riboflavin, nicotinic acid or pyridoxine either alone or in combination made little or no difference either in the rate or the density of growth. The same may be said of pantothenic acid, p-aminobenzoic acid, uracil, pimelic acid, i-inositol, and biotin methyl ester. These results indicate that some unknown factor (or factors) is required for the maximal growth of Tetrahymena, since crude casein gave far better growth than the basic medium supplemented by any or all of the known compounds mentioned above. The fact that transplantable growth occurs in the unsupplemented vitamin-free casein indicates either that the ciliates are capable of a slow synthesis of all their supplementary requirements or that the casein still contains traces of the required factors. The latter explanation seems more probable in view of the difficulty of obtaining chemically pure proteins. Therefore, until a medium of chemically known composition or one composed entirely of synthetic compounds can be formulated the question of the absolute requirement for various growth promoting substances will have to remain open.

It is clear, however, that for maximal growth the ciliates must be supplied with an outside source of unknown factors. These factors were found to be present in yeast, egg yolk, milk, timothy hay and alfalfa as well as meat (e.g. proteose-peptone). The animal sources were much lower in their content of growth promoting material than the plant sources. The former also contain a much larger proportion of protein material. For these reasons work was chiefly confined to the plant materials, especially since one of the aims was to obtain protein-free extracts of the growth promoting material in order to study the nitrogen requirements of the ciliates.

Preliminary experiments with a water extract of yeast had indicated that treatment with lead acetate gave an active precipitate. The procedure when tested on

extracts of hay or alfalfa gave precipitates which were much reduced in activity while the filtrates were usually inactive. A recombination of the two fractions gave growth very nearly equal to that of the controls (Table I). The indications are that some of the preparations may be slightly toxic, but, more important, that there are at least two factors present in hay or alfalfa which are necessary for the

TABLE I

Supplements	0	I	II	A or H
0	0	++	0	++++
I			+++ or ++++	

I—factor I
II—factor II
A—untreated alfalfa extract
H—untreated hay extract.

maintenance of growth at a maximal rate. For convenience the substance present in the material precipitated by lead acetate will be referred to as factor I and the material present in the filtrate as factor II.

It was also found that neither factor I nor factor II could be replaced by any one of the known growth supplements nor by a mixture of all the ten tested. This is further evidence for the existence of two substances of unknown structure necessary for maximum growth.

Further purification or a better separation of the two factors was attempted unsuccessfully by precipitation with lead acetate from an alkaline solution. Reprecipitation of the fractions with lead acetate was also unsuccessful, since the products gave evidence of greatly increased toxicity, possibly due to the increased phosphate concentration. In all these preparations protein was found to be present in the filtrate fraction and since both fractions are required for growth, the method is not useful for the removal of protein.

Ferric oxide hydrosol has been used as a protein precipitant. When tested on alfalfa and hay extracts it was found to behave similarly to lead acetate. There was a separation into two fractions, both required for optimum growth. The precipitate contained factor I and the filtrate contained factor II as well as protein (Table II).

The results with hay extract and ferric oxide hydrosol are similar to those given above for alfalfa extract but the separation is not so clear cut. A reprecipitation of the iron hydrosol fractions with lead acetate gave a more complete separation, but there was evidence of an increased toxicity of the fractions.

The material precipitated by sulfuric acid in preparation of the extracts for the addition of phosphotungstic acid was found to be inert whether alone or in the presence of either factor I or factor II. Upon the addition of phosphotungstic acid there was no clear separation into two active fractions and no removal of protein without appreciable loss of activity.

TABLE II

Supplements	0	I	II	FeI	FeII	A
0	0	++	+	±	±	++++
I			+++±		++++	
FeI			+++±		++++	

FeI—iron hydrosol procipitate
FeII—iron hydrosol filtrate
Other symbols as in Table I.

At this point the possibility that carbohydrate might be concerned in the activity of these fractions arose. Since all the reducing sugar in the extracts was found in the filtrate fraction from the lead acetate treatment, this fraction was treated with copper sulfate and calcium hydroxide. A complete removal of the reducing sugars but not of the protein in the preparations was possible without appreciable loss in activity. This treatment may be valuable in the further purification of the factors, since other inert materials appeared to be removed with the sugars.

Since heavy metals failed to remove protein, extractions with various organic solvents was tested as a means of obtaining protein-free preparations. Extracts prepared with ether, acetone, alcohol and acetic acid were found to be inactive or even toxic.

Dialysis also failed to remove protein or protein breakdown products. Some nitrogenous material of this nature was found to be freely diffusible as were both factor I and factor II. The dialysate in all cases was inert; all activity was found in the diffusate. The fact that there was some loss of activity from the extracts during dialysis led to the conclusion that one of the factors is destroyed by light. When the electric light bulb used to heat the box in which dialysis was carried out was screened the loss of activity did not occur. This may be correlated with the progressive loss of activity of extracts exposed to ultraviolet radiation for increasingly longer intervals (Kidder and Dewey, 1942, mistakenly state that factor I is affected by the irradiation). The results indicate that factor II is destroyed by light. This is evidence also for the organic nature of the growth promoting material.

Adsorption upon activated charcoal or Fuller's earth followed by selective elution is a well known means of purification of growth factors. When this method was tested it was found that both factors, as well as protein, are readily adsorbed upon Norit and much less readily upon Super Filtrol. The filtrate after the Norit treatment was completely inert. Both factors (as well as the protein) appear to be eluted by alkaline alcohol (Table III). The elutions from Super Filtrol were more successful, possibly because the materials are less strongly adsorbed. Although this method may be useful in the purification of the separate fractions after precipitation with lead acetate, it was discarded as a means of protein removal.

The tests of the stability of the growth substances to heat at various pH values showed that there was no loss of activity in alfalfa extracts in either acid or alkaline

TABLE III

Supplements	0	I	II	E	A
0	0	++	0	+++	++++
I			+++	++++	
II				++++	
F	+++	++++	+++±	++++	

F—filtrate after adsorption
E—Eluate from Super Filtrol.

solution. On the other hand proteose-peptone treated at an alkaline pH and used to supplement casein was almost inert. By testing it was found that this was due to a loss of factor I during the treatment (Table IV). Factor I from animal sources therefore appears to be heat-labile. The loss of activity in heat-treated proteose-peptone is not due to the destruction of thiamine. The more drastic treatment such as that described for the removal of tannins destroyed activity entirely, which is further evidence for the organic nature of the supplements.

TABLE IV

Supplements	0	I	HA	HPP	A
0	0	++	++++	±	++++
I				++++	
II	±	++++		+	
Thiamine	±	++		+	

HA—alfalfa extract heated at high pH
HPP—proteose-peptone heated at high pH.

The last method tested for the removal of protein was precipitation with organic solvents. Both the whole extracts and the fraction (filtrate) containing the protein after lead acetate precipitation were treated by the addition of alcohol up to a concentration of 75 per cent. This method was successful in the removal of protein from the hay extracts but not from the alfalfa extracts. The precipitates obtained from hay were inert and the activity of the filtrates was unaffected (Table V). Whole hay extract treated in this manner was used in the experiments on nitrogen nutrition to be described later.

The effect of the addition of barium hydroxide plus alcohol was tested on the alfalfa extract in the hope of precipitating the protein. It was found, however, that 75 per cent alcohol alone precipitated some of both factors along with some of the protein. The addition of barium then had an effect similar to that of lead acetate in that there was a partial separation of the two factors.

Acetone was next considered as a means of removing protein from alfalfa extracts. Its behavior was similar to that of alcohol in that the active substances were precipitated along with the proteinaceous material, the amount increasing as the concentration of the acetone was increased. At 80 per cent factor I was largely precipitated and factor II to a smaller extent. Since protein-free extracts could be obtained readily from hay, the work on alfalfa was discontinued even though it is a richer source of growth-promoting material.

TABLE V

Supplements	0	I	II	IIp	IIf	II	IIp	Hf
0	0	++	±	±	±	++++	±	++++
I			++++	++	++++			

IIp—precipitate from alcohol treatment of factor II fraction
IIf—filtrate from the same
Hp—precipitate from alcohol treatment of hay extract
Hf—filtrate from same.

From the above the properties of the two factors may be summarized as follows: soluble in water, moderate concentrations of alcohol and in low concentrations of acetone; insoluble in ether; stable to heat (plant sources only in the case of factor I); dialyzable through cellophane; readily adsorbed on charcoal and less readily upon Super Filtrol; eluted by ammoniated alcohol. Factor II differs from factor I in that the former is not precipitated by the salts of heavy metals and appears to be destroyed by irradiation.

When either Harris gelatin or Eastman de-ashed gelatin was used as a basic medium (1.5 per cent solution) the results obtained were similar to those obtained with casein as a basic medium, except that the population density was smaller. In the Harris gelatin alone slight but transplantable growth, which was somewhat improved upon the addition of inorganic salts, was obtained. The addition of thiamine, riboflavin, pantothenic acid or biotin gave little or no improvement in growth, while the addition of hay extract gave a considerable increase in the rate and density of growth.

With de-ashed gelatin the addition of inorganic salts was necessary and in their presence without the addition of supplements slight transplantable growth occurred. The addition of thiamine or ribroflavin (0.0001 mg. per ml.) or both together gave no improvement in growth. Growth was increased only upon the addition of both hay extract and riboflavin to the medium.

B. Nitrogenous Nutrition

The experiments to be described below are exploratory in nature and have served chiefly to suggest further experiments and modes of attack upon the problem. Some of the work of earlier investigators was repeated in the hope that the use of an adequately supplemented medium might give better results than had been obtained.

The first experiments were carried out upon completely hydrolyzed casein. Such a medium was chosen in the hope of shedding more light upon Lwoff's (1932) hypothesis that polypeptides are required for growth. Acid digestion was used because complete hydrolysis by enzymatic means is difficult if not impossible and alkaline hydrolysis has a destructive effect on many of the amino acids. No growth occurred in the acid hydrolysate even in the presence of what was considered to be adequate supplementation. Attention was then turned to a solution of pure amino acids, also supplemented with protein-free hay extract. This solution was prepared using the ten amino acids found by Rose (1938) to be necessary for the nutrition of the mammal. The amino acids were present in the concentrations found in a one per cent solution of casein. Again no growth occurred in this solution or in various dilutions of it.

Such solutions have an osmotic pressure lower than that of salt solutions readily tolerated by the organism. The explanation for the lack of growth must, therefore, be sought elsewhere. Three other possible explanations for the lack of growth are, *a*) that one (or more) amino acid required for the growth of the organism is lacking, *b*) one or more of the amino acids present is toxic or inhibitory, and *c*) that the organism requires nitrogen in the form of polypeptides.

The possibility of the toxicity of the amino acids was considered first. These experiments were to be correlated with others using gelatin as a basic medium and supplemented with one or more of the amino acids known to be lacking from this protein. For this reason those particular amino acids were added to casein as well as to gelatin in the concentrations in which they are found in a one per cent solution of casein. The results in the two media were strikingly different. With casein it was found that the addition of free amino acids had little or no effect on growth. In the case of gelatin (one per cent vitamin-free gelatin Harris) definite inhibition of growth was found in those cultures containing valine, tyrosine or isoleucine. Hydroxyglutamic acid was not then available. When tryptophane was added to the gelatin there was a large increase in the growth and media containing tryptophane in addition to valine or tyrosine gave better growth than similar media lacking tryptophane. It was found that decreasing the concentration of these amino acids to 0.0025 per cent improved the growth in all cases, although tyrosine, valine and isoleucine still showed inhibition of growth. In all cases the media contained protein-free hay extract.

These experiments were repeated in tube cultures three or four times, but in order to check the observations cultures were incubated in Kidder culture flasks and the growth followed by making counts at intervals of 12 hours. With casein plus 0.01 per cent tyrosine it was found that the population density at the end of the phase of logarithmic growth (48 hours) was 81,000 organisms per ml. and without tyrosine 75,500 per ml. The figures in the case of tryptophane were quite similar, 84,000 and 70,000 respectively, with and without 0.01 per cent tryptophane. The generation times did not differ significantly in any of the media. The differences in population density in these media represent less than one division per ciliate and are not regarded as being of statistical significance.

With one per cent gelatin as a basic medium it was found that the addition of 0.01 per cent tyrosine, valine or isoleucine gave maximum populations of only a few hundred organisms per ml. Gelatin alone gave 15,000 per ml. and with the addition of tryptophane a maximum of 90,000. When the concentration of added

amino acid was reduced to 0.0025 per cent tyrosine gave a maximum of 12,000; valine, a maximum of 7,100; isoleucine, a maximum of 900 and tryptophane a maximum of 91,000 organisms per ml. In all cases the generation time was lengthened.

When two per cent gelatin was used a population of 80,000 organisms per ml. was obtained and when 0.002 per cent tryptophane was added the maximum was 230,000 organisms per ml. In this case the amino acid caused no decrease in interdivisional time.

The above results indicate that certain amino acids are detrimental to the growth of Tetrahymena, but suggest that this inhibition is reduced or absent in the presence of large protein molecules such as casein, or in the presence of tryptophane. The growth in two per cent gelatin with and without tryptophane leads plausibility to the theory that large protein molecules or a sufficient concentration of smaller protein molecules in some way decreases the inhibitory effect of free amino acids upon the ciliates. Time did not permit the testing of the more toxic amino acids with the higher concentration of gelatin.

Silk peptone, the only other incomplete protein preparation readily available, gave such good growth when supplemented with hay extract that it was not used as a basic medium for the study of amino acid requirements.

Discussion

Of the four types of substances generally accepted as being required for growth of an organism (inorganic salts, supplementary substances, carbon and nitrogen compounds) it is evident that *Tetrahymena geleii* requires inorganic salts (Hall, 1942; Hall and Cosgrove, 1944 and data presented here), supplementary factors and an organic source of nitrogen which supplies the needs for both carbon and nitrogen. The requirement for a source of carbon separate from the source of nitrogen has never been demonstrated.

At present the question of the supplementary factor requirements of Tetrahymena remains unsettled. So far the claims that thiamine is a growth factor (i.e. an absolute requirement for growth) have not been substantiated. Indeed under certain conditions it is not even to be regarded as a growth stimulant (Kidder and Dewey, 1942). The work of Hall and Cosgrove (1944) fails to refute this claim.

Hall (1942) claims that riboflavin is also a growth factor for Tetrahymena (*Colpidium campylum*). This work could not be confirmed, although stimulation of growth could be obtained with both thiamine and riboflavin under certain conditions. In any case the growth stimulation obtained with the two unknown factors described above is far more powerful than that caused by either of these compounds.

Elliott (1935b) reports an increase in the maximum population density of cultures when pantothenic acid was added to tryptone media. Since he was using a crude preparation of pantothenic acid this effect may have been due to other substances in the preparations. Pantothenic acid has subsequently been found to have have no effect on growth when added to a casein medium. So far as can be determined from the data published (Hall, 1939; 1942) pimelic acid has no "acceleratory" effect on growth. The effect appears to be due to the introduction of inorganic salts.

Certain secondary effects have been attributed to thiamine, riboflavin and other known growth substances (Hall, 1940a; Hall and Shottenfeld, 1941; Baker and

Johnson, 1941). These are concerned with the death and decline phases of growth and are not of immediate interest here. It is of more importance to the problem under consideration that none of the known growth-promoting substances will permit maintenance of growth at the maximum rate and of a maximum density. For such growth at least two substances of unknown nature are required. Whether or not some of the known compounds may also be required for such growth cannot be decided until pure preparations of these substances and a basic medium known to contain no growth supplements are available. The use of purified gelatin for a basic medium may give information of some value, but it is not truly suitable, since its use introduces the complication that it does not satisfy a possible requirement for one or more of the amino acids it lacks. Although the so-called vitamin-free casein is not altogether ideal because it appears to contain traces of growth promoting materials, it is nevertheless an adequate basic medium for a study of growth stimulation. Unsupplemented, the growth it supports is far from maximal.

The fact that growth of these ciliates can be obtained in gelatin solutions when properly supplemented, as pointed out by Hall (1942), would indicate that the ciliate requires for growth none of the amino acids lacking from that protein. In other words *T. geleii* must synthesize tryptophane, valine, hydroxyglutamic acid, isoleucine and possibly tyrosine unless its protoplasm does not contain these amino acids. This latter hypothesis seems most unlikely especially in view of the fact that tryptophane increases the growth so remarkably. It is difficult to explain however, why tryptophane increases the maximum concentration of organisms obtained rather than the growth rate.

The failure of other investigators to obtain growth with solutions of amino acids or incomplete proteins supplemented with amino acids is now understandable. In some cases (Lwoff, 1932; Elliott, 1935b) the media contained none of the supplementary factors now known to have a profound effect on growth. Nor can the claims of Hall and Elliott (1935) regarding the effects of certain amino acids be regarded as conclusive, since their results were expressed as $x : x_0$. As Kidder (1941b) has pointed out, this method of representation may give an entirely false conception of the results obtained. Another source of possible error in the earlier work may lie in the use of concentrations of amino acids which may now be regarded as inhibitory to growth. It is possible that this difficulty may be overcome by the adsorption of amino acids upon inert colloids and by the use of tryptophane, which appears to decrease the toxicity of other free amino acids.

In view of the inhibitory effect of free amino acids and of the ability of *T. geleii* to grow in an incomplete protein such as gelatin, it is difficult to understand the report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in a solution of 15 amino acids with the addition of various supplements. It is possible that the explanation lies in the fact that different strains of *T. geleii* were used.

No definite decision can as yet be made between the three suggested possibilities for the lack of growth of *T. geleii* in amino acid solutions. The evidence on hand, however, suggests that the factor of toxicity of free amino acids is of some importance. This effect, rather than a requirement for polypeptides, is a possible explanation for the decreasing growth obtained by Lwoff (1932) as the degree of hydrolysis of the medium used was increased. This would be true whether or not adequate supplements were present.

Summary

1. The known growth promoting substances alone or in various combinations are not sufficient for the growth of *Tetrahymena geleii* at a maximal rate and density.

2. At least two unknown substances (factor I and factor II), present in both plant and animal materials, are required for such growth.

3. Factor I is distinguished from factor II by the fact that the former is precipitated by heavy metal salts while the latter is not.

4. Active protein-free preparations of these factors may be prepared from extracts of timothy hay by treatment with ethyl alcohol.

5. Growth of the ciliate could not be obtained in acid digests of casein or in solutions of free amino acids supplemented with the protein-free extract.

6. Tyrosine, valine, and isoleucine were found to be inhibitory in the presence of gelatin, but not in the presence of casein.

7. A large increase in the population density occurred in the presence of tryptophane and gelatin but not with tryptophane and casein.

LITERATURE CITED

Baker, E. G. S., and W. H. Johnson, 1941. Some effects of vitamin B components on populations of Tetrahymena geleii. *Anat. Rec.*, **81** (suppl.) : 68.

Barker, H. A., and C. V. Taylor, 1931. A study of the conditions of encystment of Colpoda cucullus. *Physiol. Zool.*, **4** : 620–634.

Claff, C. L., 1940. A migration dilution apparatus for the sterilization of protozoa. *Physiol. Zool.*, **13** : 334–341.

Dewey, V. C., 1941. The nutrition of Tetrahymena geleii (Protozoa, Ciliata). *Proc. Soc. Exper. Biol. and Med.*, **46** : 482–484.

Elliott, A. M., 1935a. Effects of certain organic acids and protein derivatives on the growth of Colpidium. *Arch. f. Protistenk.*, **84** : 472–494.

Elliott, A. M., 1935b. The influence of pantothenic acid on the growth of protozoa. *Biol. Bull.*, **68** : 82–92.

Elliott, A. M., 1939. The vitamin B complex and the growth of Colpidium striatum. *Physiol. Zool.*, **12** : 363–373.

Hall, R. P., 1939. Pimelic acid as a growth stimulant for Colpidium campylum. *Arch. f. Protistenk.*, **92** : 315–319.

Hall, R. P., 1940a. Relation of vitamins to the growth and decline of populations in Glaucoma piriformis. *Anat. Rec.*, **78** (suppl.) : 164.

Hall, R. P., 1940b. Riboflavin and thiamin as growth factors for the cilite, Colopidium campylum. *Anat. Rec.*, **78** (suppl.) : 164.

Hall, R. P., 1942. Incomplete proteins as nitrogen sources and their relation to vitamin requirements in Colpodium campylum. *Physiol. Zool.*, **15** : 95–107.

Hall, R. P., and W. B. Cosgrove, 1944. The question of the synthesis of thiamin by the ciliate, Glaucoma piriformis. *Biol. Bull.*, **86** : 31–40.

Hall, R. P., and A. M. Elliott, 1935. Growth of Colpidium in relation to certain incomplete proteins and amino acids. *Arch. f. Protistenk.*, **85** : 443–450.

Hall, R. P., and A. Shottenfeld, 1941. Maximal density and phases of death in populations of Glaucoma piriformis. *Physiol. Zool.*, **14** : 384–393.

Harrison, C. J., and E. A. H. Roberts, 1939. The fermentation process in tea manufacture. IV. Tea tannin and its fermentation products. *Biochem. Jour.*, **33** : 1408–1420.

Kidder, G. W., 1941a. Growth studies on ciliates. V. The acceleration and inhibition of ciliate growth in biologically conditioned medium. *Physiol. Zool.*, **14** : 209–225.

Kidder, G. W., 1941b. Growth studies on ciliates. VII. Comparative growth characteristics of four species of sterile ciliates. *Biol. Bull.*, **80** : 50–68.

KIDDER, G. W., AND V. C. DEWEY, 1942. The biosynthesis of thiamine by normally athiaminogenic organisms. *Growth*, **6**: 405–418.

KLINE, A. P., 1943. The nitrogen compounds necessary for growth in Colpidium striatum Stokes, with special reference to the amino acids. *Physiol. Zool.*, **16**: 405–417.

LWOFF, A., 1924. Le pouvoir de synthèse d'un protiste hétérotrophe: Glaucoma piriformis. *C. R. Soc. Biol.*, **91**: 344–345.

LWOFF, A., 1942. Recherches biochimiques sur la nutrition des protozoaires. Le pouvoir de synthèse. *Monographie de l'Institut Pasteur.*

LWOFF, A., AND M. LWOFF, 1937. L'aneurine, facteur de croissance pour le cilié Glaucoma piriformis. *C. R. Soc. Biol.*, **126**: 644–646.

LWOFF, A., AND M. LWOFF, 1938. La spécificité de l'aneurine, facteur de croissance pour le cilié Glaucoma piriformis. *C. R. Soc. Biol.*, **127**: 1170–1172.

PETERS, J. P., AND D. D. VAN SLYKE, 1931. *Quantitative clinical chemistry.* Vol. 2. Methods. The Williams and Wilkins Co., Baltimore.

ROSE, W. C., 1938. The nutritive significance of the amino acids. *Physiol. Rev.*, **18**: 109–136.

THOMAS, A. W., AND A. FRIEDEN, 1923. Ferric salts as the "solution-link" in the stability of ferric oxide hydrosol. *Jour. Amer. Chem. Soc.*, **45**: 2522–2532.